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Comparison of the Transactivation Domains of Stat5 and Stat6 in Lymphoid Cells and Mammary Epithelial Cells

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Stat (signal transducers and activators of transcription) and Jak (Janus kinases) proteins are central components in the signal transduction events in hematopoietic and epithelial cells. They are rapidly activated by various cytokines, hormones, and growth factors. Upon ligand binding and cytokine receptor dimerization, Stat proteins are phosphorylated on tyrosine residues by Jak kinases. Activated Stat proteins form homo- or heterodimers, translocate to the nucleus, and induce transcription from responsive genes. Stat5 and Stat6 are transcription factors active in mammary epithelial cells and immune cells. Prolactin activates Stat5, and interleukin-4 (IL-4) activates Stat6. Both cytokines are able to stimulate cell proliferation, differentiation, and survival. We investigated the transactivation potential of Stat6 and found that it is not restricted to lymphocytes. IL-4-dependent activation of Stat6 was also observed in HC11 mammary epithelial cells. In these cells, Stat6 activation led to the induction of the β-casein gene promoter. The induction of this promoter was confirmed in COS7 cells. The glucocorticoid receptor was able to further enhance IL-4-induced gene transcription through the action of Stat6. Deletion analysis of the carboxyl-terminal region of Stat6 and recombination of this region with a heterologous DNA binding domain allowed the delimitation and characterization of the transactivation domain of Stat6. The potencies of the transactivation domains of Stat5, Stat6, and viral protein VP16 were compared. Stat6 had a transactivation domain which was about 10-fold stronger than that of Stat5. In pre-B cells (Ba/F3), the transactivation domain of Stat6 was IL-4 regulated, independently from its DNA binding function.

Stat proteins (signal transducers and activators of transcription) are transcription factors which transmit signals from activated cytokine receptors to the nucleus. They are tyrosine phosphorylated by Jak kinases and dimerize through their SH2 domains (22, 59). A functional domain structure of the Stat proteins has been established, and the regions responsible for dimerization, specific DNA binding, and transactivation have been identified (21, 23, 49, 62). Stat5a, Stat5b, and Stat6 are the members of the Stat gene family (26) which have the highest amino acid homologies. These mammalian Stat factors are related to Marelle, a recently identified Stat protein important for *Drosophila* larval development (25, 76). The sequence homologies of Marelle with Stat5 and Stat6 are 37 and 34%, respectively.

Stat5a and Stat5b are two highly related genes expressed in most tissues (3, 31, 41, 51). Stat5a was originally found as a mediator of prolactin-induced gene transcription in the mammary gland (63, 64, 71). Prolactin controls the synthesis of milk proteins in mammary epithelial cells (4, 15, 67) but also functions in the hematopoietic system. It regulates genes important for growth and differentiation in lymphocytes (12, 79). Activation of Stat5 is not restricted to prolactin, and Stat5 has been found to respond to a wide variety of cytokines (15–17).

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Interleukin-4 (IL-4) is an effective modulator of the immune system (13, 65). It is secreted by activated T cells, thymocytes, basophils, and mast cells (54). IL-4 acts on different cell types, including lymphocytes, macrophages, monocytes, dendritic cells, keratinocytes, eosinophils, and epithelial cells (46, 65, 75). Depending on the cells involved, activation of the IL-4 receptor (IL-4R) leads to proliferation, differentiation, or cellular survival (8, 9, 20, 53, 72). In hematopoietic cells, IL-4 mediates the induction of the Th2 phenotype in T-helper cells (34, 46) and the immunoglobulin isotype switching in B cells (8, 36).

The transcriptional response to IL-4 is partly mediated through Stat6. Binding sites for Stat6 were found to correlate with phosphotyrosine residues 574 and 611 of the intracellular domain of the human IL-4R α chain (24, 53, 62). Homodimerization of IL-4R α chains is sufficient for Jak1 and Stat6 activation in Ba/F3 cells (28). The high-affinity IL-4R complex of lymphocytes consists of the IL-4R α chain (45) and the common γ_c chain, which is also a structural component of the IL-2, IL-7, IL-9, IL-12, and IL-15 receptors (33, 37, 52). Human Stat6 (h-Stat6) and mouse Stat6 genes have 83% amino acid identity (24, 48). The biological role of Stat6 in IL-4 signalling was shown in mice in which the Stat6 gene was inactivated by homologous recombination (29, 66, 69). Mice deficient in Stat6 had defects similar to those of mice lacking the IL-4 gene (34, 36, 70). Deficient mice did not develop Th2 T-helper cells and had low surface expression levels of CD23, IL-4R α, and major histocompatibility complex class II molecules and low levels of

immunoglobulin E in their sera. Therefore, these mice were highly susceptible to helminthic parasites.

The carboxyl-terminal regions of the Stat factors show the most pronounced sequence diversity. Studies of naturally occurring splice variants of Stat1, Stat3, Stat5a, and Stat5b and of carboxyl-terminal deletion mutants of Stat1, Stat2, Stat3, Stat5a, and Stat5b (30, 31, 44, 47, 49, 51, 58, 60) have shown that the carboxyl-terminal domain is essential for gene transcription. The transactivation potential of Stat6 was tested by the introduction of a Stat6 response element-containing promoter gene construct into IL-4-responsive cells (8, 32).

In this study, we characterized the carboxyl-terminal region of Stat6, which is required for efficient transactivation. We compared the transactivation properties of Stat5 and Stat6 in different cell types. The β-casein, cytokine inducible sequence (CIS), and oncostatin M (OSM) promoters were used to measure transcriptional induction. These promoters contain adjacent Stat response elements (64, 77, 78). Prolactin induces β-casein gene transcription through the action of Stat5 in COS7 and HC11 cells. Stat6 is also able to induce β-casein expression through binding to the Stat sites in the promoter region. We compared the transactivation efficacy of Stat5 with those of Stat6 and the acidic transactivator VP16.

MATERIALS AND METHODS

Cell cultures and transfections. HC11 cells were grown in RPMI medium containing 2 mM glutamine, 10% fetal calf serum (FCS), 5 μg of bovine insulin (Sigma) per ml, and 10 ng of murine epidermal growth factor (Sigma) per ml and were stably transfected with promoter constructs as previously described (67). Hormone induction was performed by the addition of 0.1 mM dexamethasone (Sigma) and/or 5 μg of ovine prolactin (Sigma) per ml and in the absence of epidermal growth factor in competent cultures. HC11 cells were induced with 5% culture supernatant of murine IL-4-overproducing cells. COS7 cells were maintained in Dulbecco modified Eagle medium containing 10% FCS and 2 mM glutamine and were transfected and induced as described previously with prolactin (16) or 100 U of human IL-4 per ml. Activation of the glucocorticoid receptor (GR) was performed by 10^{-7} M dexamethasone treatment for 16 h as previously published (68). The murine pre-B-cell line Ba/F3, stably transfected with the prolactin receptor (12), was cultured in RPMI medium containing 2 mM glutamine, 10% FCS, and 10 ng of ovine prolactin per ml. Ba/F3 cells, stably transfected with the human IL-4R α and γ chains, were cultured in RPMI medium containing 2 mM glutamine, 10% FCS, and 5% culture supernatant of murine IL-3-overproducing cells as previously described (40). Before induction with ovine prolactin or human IL-4, cells were maintained in medium with 3% horse serum for 3 h and then stimulated with 1 μg of prolactin per ml or 100 U of IL-4 per ml for the times indicated. COS7 cells were transfected by the calcium phosphate method. Chloramphenicol acetyltransferase (CAT), luciferase, and β-galactosidase activities were measured as previously described (67, 71). Two micrograms of expression vector, 2 μg of the luciferase constructs, and 1 μg of pCH110 were used in transfection experiments. Ba/F3 cells (6×10^6) stably transfected with the prolactin receptor or human IL-4R chains were electroporated for each point with a Bio-Rad gene pulser at 250 V/960 μF. Electroporated Ba/F3 cells were first cultured in growth medium, and 4 h later, cells were washed and unstimulated cells were maintained in medium containing 10% FCS. Stimulated cells were induced with prolactin or IL-4. Ba/F3 cells have high levels of endogenous β-galactosidase; therefore, the total protein amount was measured for normalization of luciferase activity. After 24 h, electroporated cells were harvested.

Plasmids. (i) Stat5, Stat6, GR, cytokine receptor chain, and luciferase reporter constructs. The reporter gene constructs containing the β-casein gene promoter (−344 to −1) linked to the CAT gene or the luciferase gene have previously been described (10, 71), as have the expression vectors for mammary gland factor (MGF)-Stat5a (pXM-MGF), human GR (pRSVhGRα), and the long form of the murine prolactin receptor (44, 68, 71). pCH110 encodes the β-galactosidase gene under the control of the simian virus 40 promoter. The expression vectors for the human IL-4R α and γ_c chains have been described previously (40). The expression vector for h-Stat6 (pXM-Stat6) was subcloned as an *Eco*RI-*Kpn*I fragment (24), and the 5′ untranslated region between the unique *Eco*RI and *Nhe*I sites was deleted. The construct encoding h-Stat6Δ677 was derived by deletion of the region between the unique *Sac*I (nucleotide 1978) and *Xho*I (adjacent to the *Kpn*I site within the 3′ untranslated region) sites of pXM-Stat6 and ligation to a PCR-generated fragment cut with *Sac*I and *Xho*I. The PCR-generated fragment was generated with an upper primer which comprised nucleotides 1912 to 1930 and a lower primer which comprised a unique *Xho*I site for h-Stat6Δ677 introduced adjacent to the stop codon (nucleotides 2029 to 2031 [gat→tga]).

The minimal promoter construct N3(Stat-RE)6-tk-luc was described previously (44). The multimerized Stat6 response element N4(Stat-RE)3 from the immunoglobulin germ line heavy-chain ε promoter was subcloned from a CAT vector (kindly provided by Albert Duschl) as a *Sph*I-*Xba*I fragment, which was subcloned in front of the thymidine kinase (TK) minimal promoter driving the luciferase gene. N4(Stat-RE)3 comprises the following sequence: 5′-CATCGACTTCCCAAGAACAGAATCGACTTCCCAAGAACAGAATCGACTTCCCAAGAACAGT-3′ and 3′-GTACGTAGCTGAAGGGTTCTTGTCTTAGCTGAAGGGTTCTTGTCTTAGCTGAAGGGTTCTTGTCAGATC-5′. The reporter gene constructs containing the murine OSM gene promoter (−662 to +48) or the CIS gene promoter (−646 to −1) linked to the luciferase gene were kindly provided by A. Miyajima (Tokyo, Japan) and have partly been described elsewhere (77, 78).

(ii) GAL4 DNA binding domain constructs. Expression vectors for GAL4, GAL4-VP16, and (GAL4)×3-tk-luc were provided by R. Schüle (Freiburg, Germany). The GAL4-Stat5a-722-794 construct was described previously (44). Stat6 carboxyl-terminal fragments were PCR generated and cloned into the expression vector encoding the DNA binding domain of GAL4 (amino acids 1 to 147) (56). cDNA fragments of h-Stat6 were PCR generated with downstream primers comprising a unique *Eco*RI site and upstream primers comprising unique *Bam*HI sites flanking amino acid positions 642 to 847, 677 to 847, 705 to 847, 754 to 847, 792 to 847, 677 to 791, 677 to 704, and 754 to 791. The GAL4-VP16 construct contained amino acids 411 to 489 of VP16 (55).

(iii) Stat5-Stat6 transactivation domain chimeras. The expression vector for MGF-Stat5aΔ750 (44) was used as the basis for Stat5-Stat6 transactivation domain chimeras. A PCR fragment was created to introduce a unique *Eco*RI site at amino acid 750. The PCR fragment was generated with an upper primer which comprised nucleotides 1825 to 1847 and a lower primer which comprised a unique *Eco*RI site adjacent to amino acid 750 of MGF-Stat5. The PCR fragment was subcloned as a *Kpn*I (nucleotide 1837)-*Eco*RI fragment into pXM-Stat5aΔ750 and ligated with the PCR-generated GAL4 fusion proteins described above, each of which contained a unique *Eco*RI site at the 5′ end and a unique *Bam*HI site adjacent to the stop codon.

The correct nucleotide sequences of PCR products and cloning junctions were verified by DNA sequencing with a dideoxy T7 DNA sequencing kit (Pharmacia).

Antibodies and immunoblotting analysis. Antisera directed against the carboxyl-terminal domain of Stat6 (amino acid positions 633 to 837) were generated in rabbits and chickens. These antisera were used in supershift experiments and tested against all recombinant Stat proteins. They recognized specifically Stat6 from mice, rats, and humans. The antiserum against the SH2 domain of mouse Stat6 (amino acid positions 523 to 659) was generated in rabbits and has no cross-reactivity to any other Stat family member. Protein fragments were produced in bacteria as maltose-binding protein fusion proteins according to the manufacturer's (New England Biolabs) protocol. The phosphotyrosine-specific antibody (PY20) was purchased from Transduction Laboratories. A mouse monoclonal antibody (Santa Cruz), directed against the DNA binding domain of GAL4, was used for supershift experiments to detect GAL4-specific DNA binding activity. The polyclonal chicken antiserum against the N-terminal portion of MGF-Stat5a was previously described (71). The polyclonal rabbit antiserum against the β-casein protein was previously described (10). Immunoreactive bands were visualized with an epichemiluminescence Western blotting system (Amersham, Braunschweig, Germany) according to the manufacturer's protocol.

Preparation of cytosolic and nuclear extracts and electrophoretic mobility shift assays. For cytosolic extracts from cell culture, 10^7 cells were washed twice with phosphate-buffered saline, scraped, and lysed in hypotonic buffer containing 20 mM HEPES (pH 7.6), 10 mM KCl, 1 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.1% Triton X-100, 20% glycerol, 5 μg of aprotinin per ml, 5 μg of leupeptin per ml, 2 μg of pepstatin per ml, and 2 mM phenylmethylsulfonyl fluoride by 20 strokes in a glass Dounce homogenizer. The extract was centrifuged for 5 min at 2,000 × *g*, and the supernatant was used. Whole-cell extracts for bandshift and Western blotting were prepared by suspension of cell pellets in a buffer containing 400 mM NaCl, 50 mM KCl, 20 mM HEPES (pH 7.9), 1 mM EDTA, 20% glycerol, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 5 μg of leupeptin per ml, 5 μg of aprotinin per ml, 100 μM sodium orthovanadate, and 20 μM phenyl arsine oxide. Freeze-thawing was repeated three times, and the lysates were centrifuged for 15 min at 4°C and 20,800 × *g*. The supernatants were recovered for bandshift and Western blotting experiments. The protocol for bandshift assays has previously been described (15). In bandshift experiments, the MGF-Stat5 binding site of the bovine β-casein promoter, 5′-AGATTTCTAGGAATTCAAATC-3′, was used as the probe. This oligonucleotide was end labelled with polynucleotide kinase to a specific activity of 8,000 cpm/fmol. The sequence of the oligonucleotide used to detect GAL4-specific DNA binding activity is 5′-GATCGCACAGTGCCGGAGGACAGTCCTCCGGTTCGAT-3′ (the GAL4 binding motif is underlined). This oligonucleotide was labelled with Klenow enzyme to a specific activity of 8,000 cpm/fmol. A double-stranded annealed oligonucleotide with AT overhangs, 5′-TATGTACCCCTACGACGTCCCCGACTACGCCTCAGG-3′, was used at 0.05 μg/μl to block unspecific binding and to detect GAL4-specific DNA binding activity.

Sequence comparison. Sequence alignments were performed by using PILEUP from the Wisconsin package of the Genetics Computer Group (Version

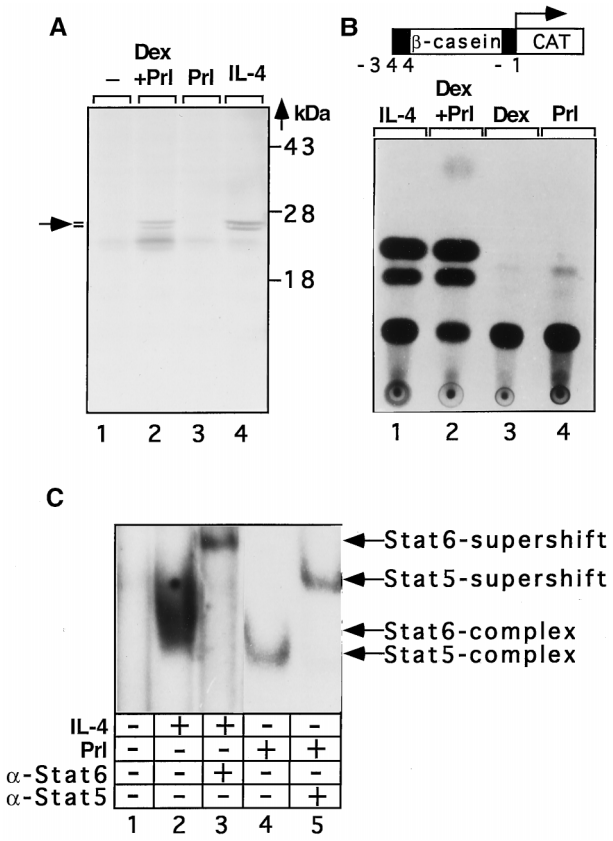


FIG. 1. Lactogenic-hormone induction of Stat5 and IL-4 induction of Stat6 activate β-casein gene transcription in mammary epithelial cells. (A) HC11 mammary epithelial cells were treated with no hormones (−; lane 1), dexamethasone, and prolactin (Dex + Prl) (lane 2), prolactin (lane 3), or IL-4 (lane 4). Whole-cell protein extracts were prepared, separated by gel electrophoresis, and analyzed by Western blotting with a mouse β-casein-specific antiserum. The arrow and horizontal lines indicate the positions of differently phosphorylated forms of β-casein. (B) HC11 cells stably transfected with a β-casein promoter–CAT construct were treated with IL-4 (lane 1), dexamethasone and prolactin (lane 2), dexamethasone (lane 3), or prolactin (lane 4). Four independent experiments were carried out, with similar results. (C) Bandshift assays of Stat6 activated (+) by IL-4 (lanes 2 and 3) and Stat5 activated by prolactin (lanes 4 and 5). Whole-cell extracts were prepared and introduced in bandshift assays. The proximal β-casein promoter Stat binding site served as the probe. Supershifts were carried out with (+) a polyclonal antiserum directed against Stat6 (633 to 837) (α-Stat6; lane 3) or Stat5 (α-Stat5; lane 5).

8.1). Sequences with the following GenBank accession numbers were used: h-Stat6, A54740; murine Stat6, L47650; murine OSM, D31942; and murine CIS, D31943.

RESULTS

β-Casein gene transcription in mammary epithelial cells is induced by lactogenic hormones via Stat5 or by IL-4 via Stat6. The induction of milk protein expression in the cultured mammary epithelial cell line HC11 (4) requires the synergistic action of lactogenic hormones, glucocorticoids, prolactin, and insulin (4, 10). Prolactin contributes through activation of transcription factor Stat5, and glucocorticoid contributes hormone through activation of the GR. Stat5 and the GR act synergistically in efficient induction of transcription (68).

We analyzed β-casein gene expression in HC11 cells grown in the presence of insulin and induced with prolactin and glucocorticoids. Cytosolic cell extracts were prepared from induced cells and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and β-casein was visualized with a specific antiserum (Fig. 1A, lane 2). The doublet around 24 kDa is characteristic for the differentially phosphorylated forms of β-casein in HC11 cells. The faster-migrating band represents a dexamethasone-inducible milk protein which is also recognized by the antiserum used. The induction of β-casein expression occurs at the transcriptional level. HC11 cells stably transfected with a β-casein promoter–CAT construct were efficiently induced only by dexamethasone and prolactin (Fig. 1B, lane 2), not by prolactin (lane 4) or dexamethasone alone (lane 3).

The promoter region used in this experiment comprises the sequences −344 to −1 relative to the transcription start site (Fig. 1B). This region includes two recognition elements of dyad symmetry that are indicative of Stat protein binding (TTC-GAA). One element is located between −144 and −134; its sequence is 5′-TTCTTGGGAA-3′. The second element is located between −97 and −89; its sequence is 5′-TTCTTGG AA-3′. Both elements are capable of binding to Stat5; the upstream element has a lower affinity (63). A comparative analysis of Stat proteins showed that Stat6 binding to DNA was favored when the symmetric half sites were separated by 4 nucleotides (62), as found in the distal element. The inducibility of β-casein in some cytotoxic T-cell lines by IL-4 has been reported previously (19). Consistent with this observation, we found a functional IL-4R complex in mammary epithelial cells. These observations led us to investigate the inducibility of the β-casein gene promoter by the activation of Stat6 in HC11 cells. β-Casein was induced by IL-4 (Fig. 1A, lane 4), and this induction was transcriptional and conferred by the −344-to-−1 promoter element (Fig. 1B, lane 1). Glucocorticoid hormone was not required. The extents of induction of the endogenous β-casein gene and of the β-casein gene promoter construct by IL-4 or prolactin and glucocorticoid hormone were similar.

Bandshift assays were carried out to investigate the ability of Stat6 to bind to the Stat response elements found in the β-casein gene promoter. When the proximal binding element was used as a probe (Fig. 1C), we observed that Stat5, as well as Stat6, was able to bind to this sequence. Nuclear extracts from HC11 cells induced with IL-4 formed a specific DNA complex with the radioactive DNA probe (Fig. 1C, lane 2). This complex contains Stat6, since it can be supershifted with a Stat6-specific antiserum (Fig. 1C, lane 3). Stat5 induced by prolactin was also able to bind to this element (Fig. 1C, lanes 4 and 5), as shown previously (15, 71). Stat6 from HC11 cells activated by IL-4 was able to bind the immunoglobulin heavy-chain ε response element and the β-casein promoter-proximal element equally well (data not shown).

IL-4-induced transcription of the β-casein gene promoter in transfected COS7 cells. To further study IL-4 activation of Stat6 and the transactivation potential of this factor, transient-transfection experiments with COS7 cells were carried out. These cells do not endogenously express Stat6 but contribute all the components necessary for IL-4 signalling upon provision of Stat6. Therefore, it is possible to study Stat6 variants in the absence of the wild-type molecule. We have previously used this approach to study prolactin action and Stat5 function (15, 44).

COS7 cells were transiently transfected with the IL-4R α and γ_c chains, wild-type h-Stat6 (Fig. 2A), and the β-casein gene promoter–luciferase reporter construct (Fig. 2). The induction of transiently transfected COS7 cells with IL-4 resulted in the expression of h-Stat6 (Fig. 2B, lanes 1 and 2), tyrosine phosphorylation of h-Stat6 (Fig. 2B, lanes 1 and 2), and specific DNA binding of the molecule (Fig. 2C, lanes 1 and 2), which can be supershifted by Stat6-specific antiserum (Fig. 2C, lane 3). In addition, the induction (eightfold) of the β-casein gene

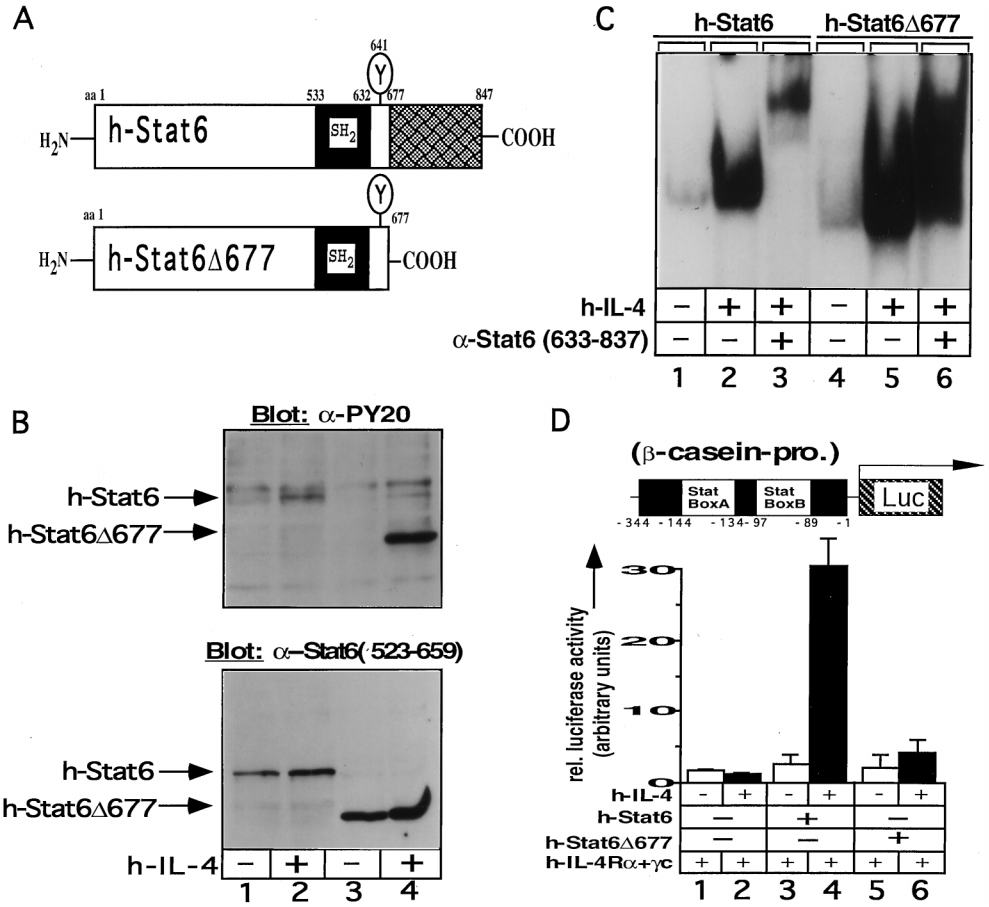


FIG. 2. Stat6 activation and β-casein promoter–luciferase induction by IL-4 in transfected COS7 cells. (A) Structures of human-Stat6 and the carboxyl-terminal deletion mutant h-Stat6Δ677. Amino acid (aa) positions, the SH2 domain, and the cytokine-dependent phosphorylation site (Y) are indicated. (B) COS7 cells were transfected with h-Stat6, h-Stat6Δ677, IL-4R α and γ_c chains, a β-casein–luciferase gene, and the β-galactosidase gene. Cells were untreated (−) or treated (+) with IL-4 for 1 h. Extracts were prepared and separated by gel electrophoresis. Tyrosine phosphorylation of h-Stat6 and h-Stat6Δ677 was probed with tyrosine phosphate-specific antiserum (α-PY20). Expression of h-Stat6 and h-Stat6Δ677 was determined with Stat6-specific antiserum [α-Stat6(523-659)]. (C) DNA binding activities of h-Stat6 and h-Stat6Δ677. Whole-cell extracts were prepared and introduced in bandshift assays. The proximal β-casein promoter Stat binding site served as the probe. Supershift experiments were carried out with (+) or without (−) Stat6-specific antiserum [α-Stat6(633-837)]. (D) Transactivation of a β-casein promoter (β-casein-pro.)–luciferase (Luc) construct by h-Stat6 and h-Stat6Δ677. Transfected cells were untreated (−) or treated (+) with IL-4, cell extracts were prepared, and relative (rel.) luciferase activities were determined. The β-galactosidase gene was included in each transfection to monitor transfection efficiency. The data obtained were normalized against β-galactosidase activities. Four independent experiments were carried out.

promoter–luciferase reporter construct was observed (Fig. 2D, lanes 3 and 4). A β-galactosidase gene was included in each transfection, and β-galactosidase activity served as a control for the transfection efficiency. We expressed the relative luciferase activity as the luciferase-to-galactosidase activity ratio.

We also derived a carboxyl-terminal deletion mutant of h-Stat6 to investigate the role of this region in transactivation. A stop codon was introduced at amino acid position 677 (h-Stat6Δ677) (Fig. 2A). Upon transfection into COS7 cells and IL-4 treatment, h-Stat6Δ677 expression (Fig. 2B, lanes 3 and 4), tyrosine phosphorylation (Fig. 2B, lanes 3 and 4), and specific DNA binding (Fig. 2C, lanes 4 and 5) were observed; h-Stat6Δ677 can be only weakly supershifted. It lacks the amino acids, 678 to 847, against which the antiserum was made (Fig. 2C, lane 6). Both h-Stat6 and h-Stat6Δ677 were able to bind to the proximal and distal β-casein promoter Stat binding sites as well as the Stat6 binding site identified in the immunoglobulin ε promoter. The β-casein promoter element and the immunoglobulin ε site have a spacing of 4 bp that separates the palindromic sequence 5′-TTCN_4GAA-3′ (data not shown). In contrast to the wild-type h-Stat6 molecule, h-Stat6Δ677 was not able to support transcriptional induction of the β-casein gene promoter–luciferase reporter construct (Fig. 2D, lanes 5 and 6).

Stat6 cooperates with the GR in the induction of the β-casein gene promoter. We have previously shown that Stat5 and the GR functionally interact in the regulation of the β-casein gene promoter. Since the extent of induction is dependent upon this interaction, we investigated whether such cooperation could also be observed between Stat6 and the GR. COS7 cells were transiently transfected with h-Stat6, the GR, and the β-casein gene promoter–luciferase reporter construct (Fig. 3). The induction of the β-casein gene promoter–luciferase gene was observed upon IL-4 treatment (Fig. 3, lane 2). Luciferase activity was four times higher upon dexamethasone and IL-4 induction. The induction of the GR alone in the presence of IL-4 had no effect on the luciferase gene (Fig. 3, lane 6). The introduction of the h-IL-4R α chain, the common γ_c chain, or both chains into COS7 cells did not change the inducibility observed (Fig. 3). We recently detected that the functional IL-4–IL-13 receptor system is endogenously expressed in COS cells (43a).

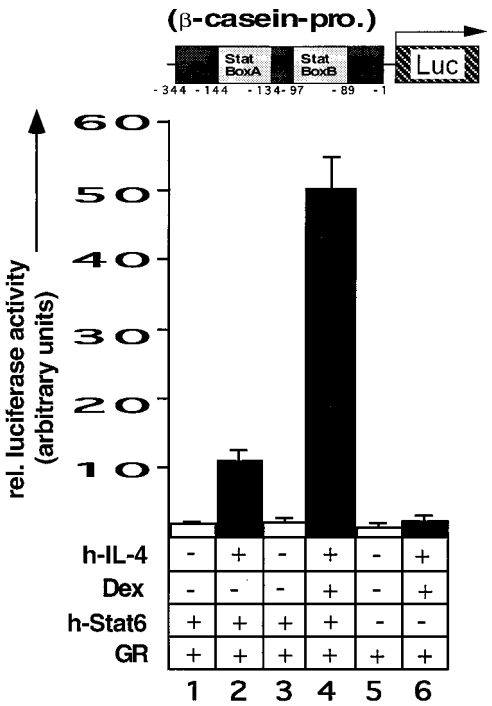


FIG. 3. Dexamethasone (Dex) induction of the GR and IL-4 induction of Stat6 result in cooperation in the activation of β-casein gene transcription. COS7 cells were transfected with h-Stat6, the GR, a β-casein promoter (β-casein-pro.)–luciferase (Luc) gene, and the β-galactosidase gene. Transfected cells were treated with (+) or without (−) IL-4 or dexamethasone, cell extracts were prepared, and relative (rel.) luciferase activities were determined. The β-galactosidase gene was included to monitor transfection efficiency. The data obtained were normalized against β-galactosidase activities. Three independent experiments were carried out.

The carboxyl terminal domain of Stat6 contains a strong, autonomous transactivation domain. As described above, we observed that deletion of the carboxyl-terminal 170 amino acids of Stat6 resulted in the loss of transcriptional induction. To investigate whether this region corresponds to the transactivation domain of the molecule, we constructed chimeric proteins consisting of the DNA binding domain of the yeast transcription factor GAL4 (56) and segments of the carboxyl-terminal region of h-Stat6 (Fig. 4A). The acidic transactivation domain of VP16 was used as a positive control (55). The transactivation domain of Stat5a was used for comparison (44).

The chimeric genes were cloned into expression vectors and transfected into COS7 cells. The expression of GAL4-Stat6 fusion proteins was analyzed by Western blotting with a GAL4-specific antibody. Fusion proteins of the expected sizes were detected (data not shown). Whole-cell extracts were prepared from transfected cells and analyzed in bandshift assays. The specific DNA sequence recognized by GAL4 was used as the probe. All fusion proteins were able to bind to the palindromic GAL4 site (Fig. 4B, lanes 3 to 24). The specificity of binding was examined in supershift assays with an antibody specific for the GAL4 DNA binding domain (Fig. 4B, even-numbered lanes).

The transactivation potential of GAL4-Stat6 fusion proteins were tested. A luciferase reporter construct containing three GAL4 binding sites in its promoter region (Fig. 4C) was cotransfected with GAL4-Stat6 fusion proteins and a β-galactosidase gene into COS7 cells. Luciferase and β-galactosidase activities were measured, and the ratios of the two activities were plotted (Fig. 4C). GAL4-642-847 (Fig. 4C, lane 3) and GAL4-677-847 (lane 4) showed 15-fold-higher activities than that of the GAL4 control (lane 4); the activities of GAL4-705-847 and GAL4-754-847 were about 7-fold higher than that of GAL4 (lanes 5 and 6). No significant transactivation was detected when GAL4-792-847 (Fig. 4C, lane 7), GAL4-677-704 (lane 9), or GAL4-754-791 (lane 10) was employed. GAL4-677-791 retained considerable transactivation potential (Fig. 4C, lane 8). Very strong transactivation (275-fold) was observed when the GAL4 DNA binding domain was fused to VP16 (Fig. 4C, lane 11). Only weak transactivation (threefold) was observed for the GAL4-Stat5a-722-794 fusion protein.

The transactivation domain of Stat6 functions efficiently in lymphoid cells. IL-4-dependent gene regulation mediated by Stat6 was originally studied with B and T lymphocytes, and inducible gene promoters were characterized. Stat factor activation in lymphocytes is not restricted to Stat6; Stat5 can be activated in these cells by cytokines like IL-2, IL-3, or prolactin (16, 17). It is conceivable that the transactivation potential of a Stat factor is cell type specific. For this reason, we compared the transactivation domains of Stat5 and Stat6 in Ba/F3 cells. Ba/F3 cells are pre-B cells which depend on IL-3 for growth. For our experiments, we used a Ba/F3 cell line stably transfected with the mouse prolactin receptor (Ba/F3-PR) (12). These cells can grow in prolactin or IL-3. Both cytokines activate Stat5 but not Stat6, in this cell line (44).

The transactivation potentials of GAL4 fusion constructs were tested by cotransfection of a luciferase reporter construct containing three GAL4 binding sites in its promoter into Ba/F3-PR cells (Fig. 5). GAL4-642-847, GAL4-677-847, and GAL4-705-847 caused a >100-fold induction of luciferase activity (Fig. 5, lanes 7 to 12), and GAL4-754-847 and GAL4-677-791 caused about 70-fold induction (lanes 13, 14, 17, and 18). Very weak induction was observed when GAL4-792-847 (Fig. 5, lanes 15 and 16), GAL4-677-704 (lanes 19 and 20), and GAL4-754-791 (lanes 21 and 22) were used. These results are consistent with the ones observed for transfected COS7 cells.

When we compared the transactivation domains of Stat5a and VP16 with that of Stat6, cell-type-specific effects were found. GAL4-Stat5a-722-794 showed only threefold induction of luciferase activity (Fig. 5, lanes 5 and 6), and GAL4-VP16 showed 60- to 100-fold induction (lanes 23 and 24). Transactivation by GAL4-VP16 is slightly lower in Ba/F3-PR cells than is transactivation by the optimal Stat6 constructs (GAL4-642-847, GAL4-677-847, and GAL4-705-847). This is in contrast to the data obtained with COS7 cells, where GAL4-VP16 caused 10-fold-higher transactivation than did the optimal Stat6 constructs. No cell-type-specific effects were observed for the transactivation domain of Stat5.

The treatment of transfected cells with prolactin had no significant effect on luciferase activities. We conclude that Stat6 contains a transactivation domain which functions with different efficiencies in COS7 and Ba/F3 cells. Stat5a contains a transactivation domain which functions only weakly in COS7 and Ba/F3-PR cells (44). The transactivation domains of Stat5a and Stat6 are not influenced by growth-promoting conditions in Ba/F3-PR cells, i.e., the action of prolactin.

The transactivation domain of h-Stat6 independently of its DNA binding domain, is IL-4 inducible in lymphoid cells. The DNA binding function of Stat6 is activated by IL-4 through phosphorylation on tyrosine 641 (43). We investigated whether the transactivation function of Stat6 is constitutively active or this function is also influenced by IL-4R activation in Ba/F3 cells. For this purpose, we used a Ba/F3 cell line stably expressing the human IL-4R α and γ_c chains (Ba/F3-IL4R) (40).

Mouse IL-4, via the endogenous IL-4R, and human IL-4, via the transfected human receptor, caused the induction of spe-

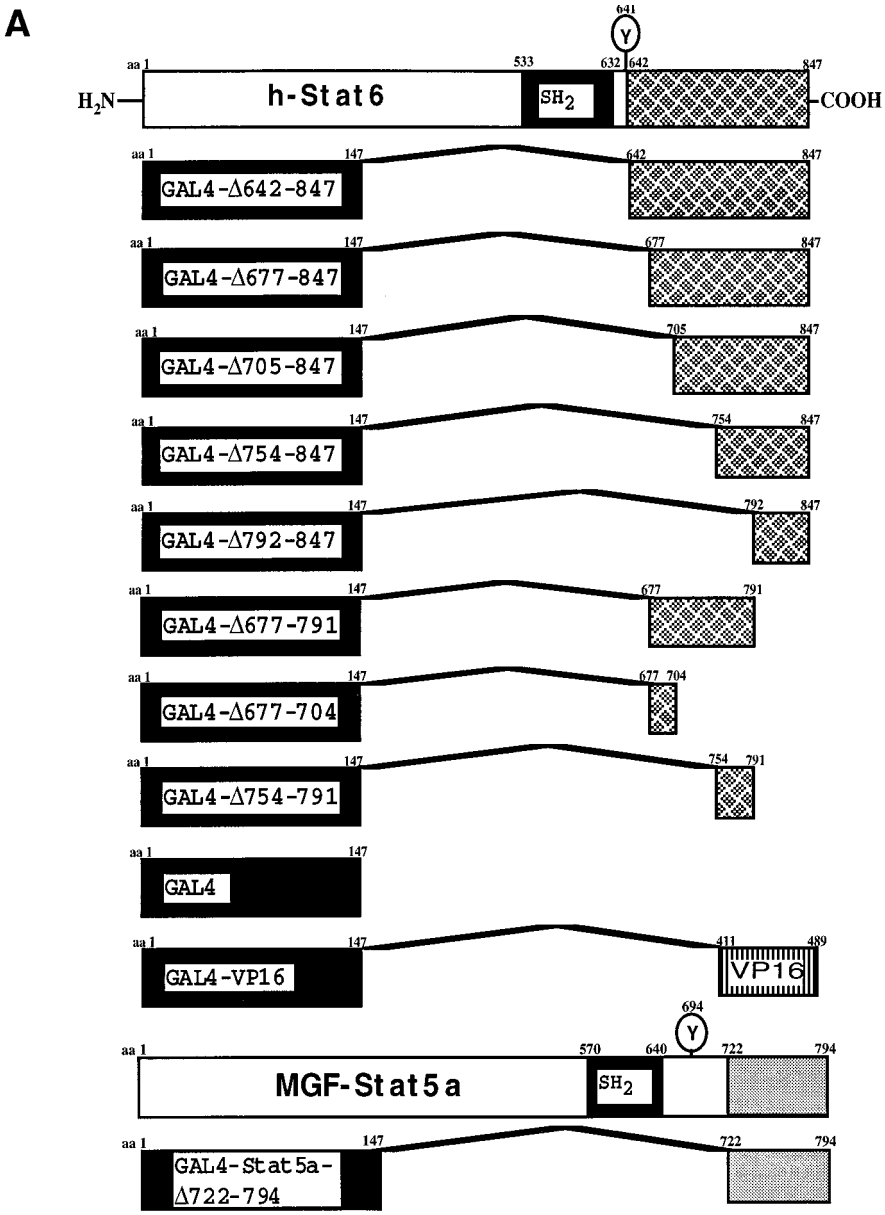
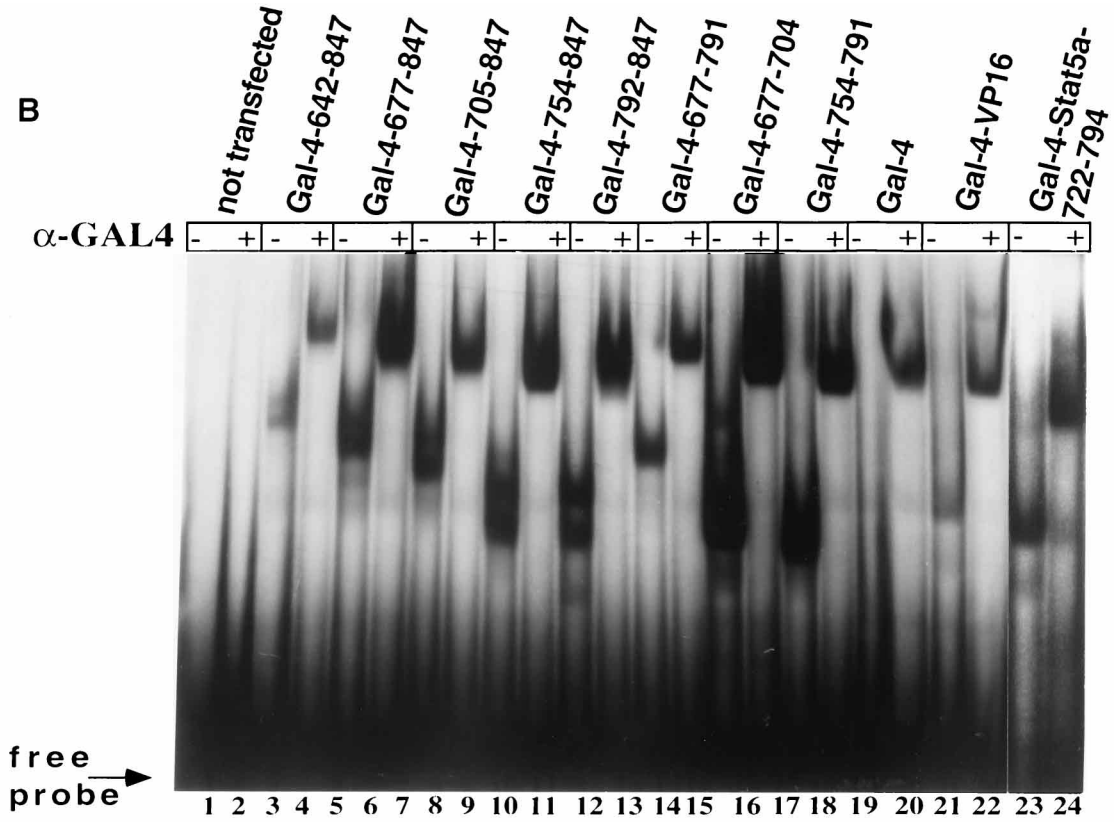


FIG. 4. Expression, DNA binding, and transcriptional induction of GAL4-Stat6 transactivation domain fusion proteins. (A) Structures of GAL4-Stat6 fusion proteins. The DNA binding and dimerization domain of yeast GAL4 (amino acids [aa] 1 to 147) is shown on the left, and the regions of h-Stat6 or MGF-Stat5a are shown on the right. The names of constructs and the amino acid positions of fused regions are indicated. The phosphorylation site (Y) is also indicated. (B) DNA binding activities of GAL4 and GAL4-Stat6 and GAL4-Stat5a fusion proteins. COS7 cells were transfected with fusion constructs. Whole-cell extracts were prepared and introduced in bandshift assays. The GAL4-specific binding site served as the probe. Supershift experiments with (+) or without (−) a monoclonal antibody directed against the DNA binding domain of GAL4 (α-GAL4) were carried out. (C) Transactivation by GAL4, GAL4-Stat6, GAL4-VP16 and GAL4-Stat5a. Fusion genes were cotransfected with a GAL4-reporter construct [$(Gal4\text{-}RE)_3$] into COS7 cells. The β-galactosidase gene was included to monitor transfection efficiency. Relative (rel.) luciferase activities were determined and normalized with β-galactosidase activities. Each luciferase activity is expressed as fold activation with respect to that of the GAL4-luciferase (Luc) reporter construct in the absence of cotransfected factors. Five independent experiments were carried out.

cific Stat6-DNA complexes (Fig. 6A, lanes 2 and 3). The identities of IL-4-induced complexes were verified by supershift experiments with Stat6-specific chicken antiserum, directed against Stat6(633-837) (Fig. 6A, lanes 4 and 5). We investigated human IL-4 induction and activation of Stat6 in Ba/F3-IL4R cells. For this reason, we constructed a minimal promoter element with three copies of the Stat6 response element from the immunoglobulin ε promoter linked to the TK promoter and a luciferase gene [N4(Stat-RE)3-tk-luc] (Fig. 6B). The dyad Stat6 half sites are separated by 4 nucleotides. Introduction of the N4(Stat-RE)3-tk-luc reporter construct in Ba/F3-IL4R cells by electroporation led to fivefold induction upon IL-4 treatment (Fig. 6B, lanes 1 and 2). This induction was further enhanced when h-Stat6 was cotransfected (Fig. 6B, lanes 3 and 4).

We investigated the possibility that the induction of Stat6 by IL-4 not only is dependent on regulation of the Stat6 DNA binding activity but also is mediated by control of the transactivation domain. GAL4, GAL4-Stat6-677-791, GAL4-VP16, and the (GAL4-RE)3-tk-luc reporter construct were tran-



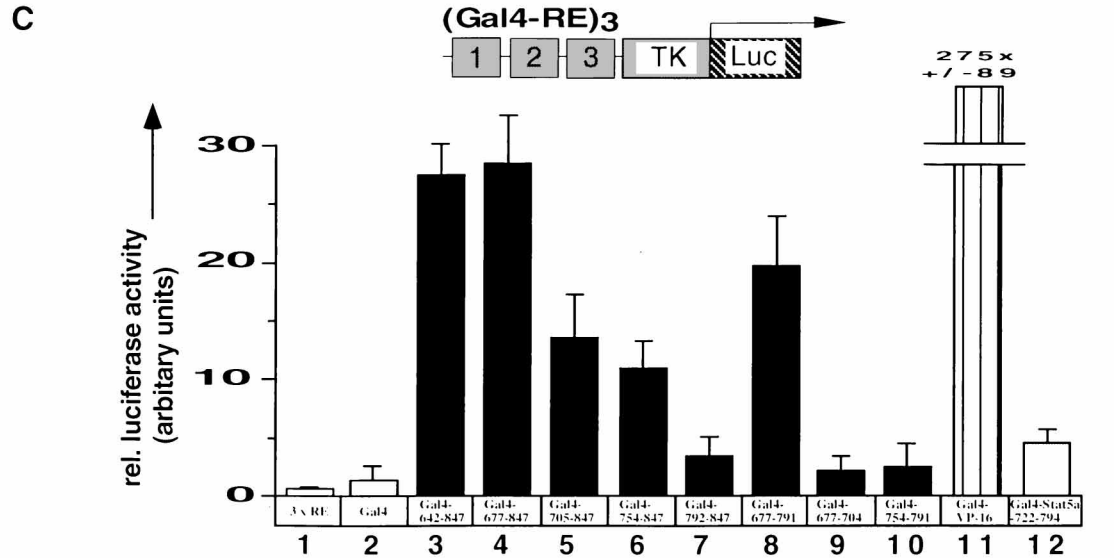


FIG. 4—*Continued.*

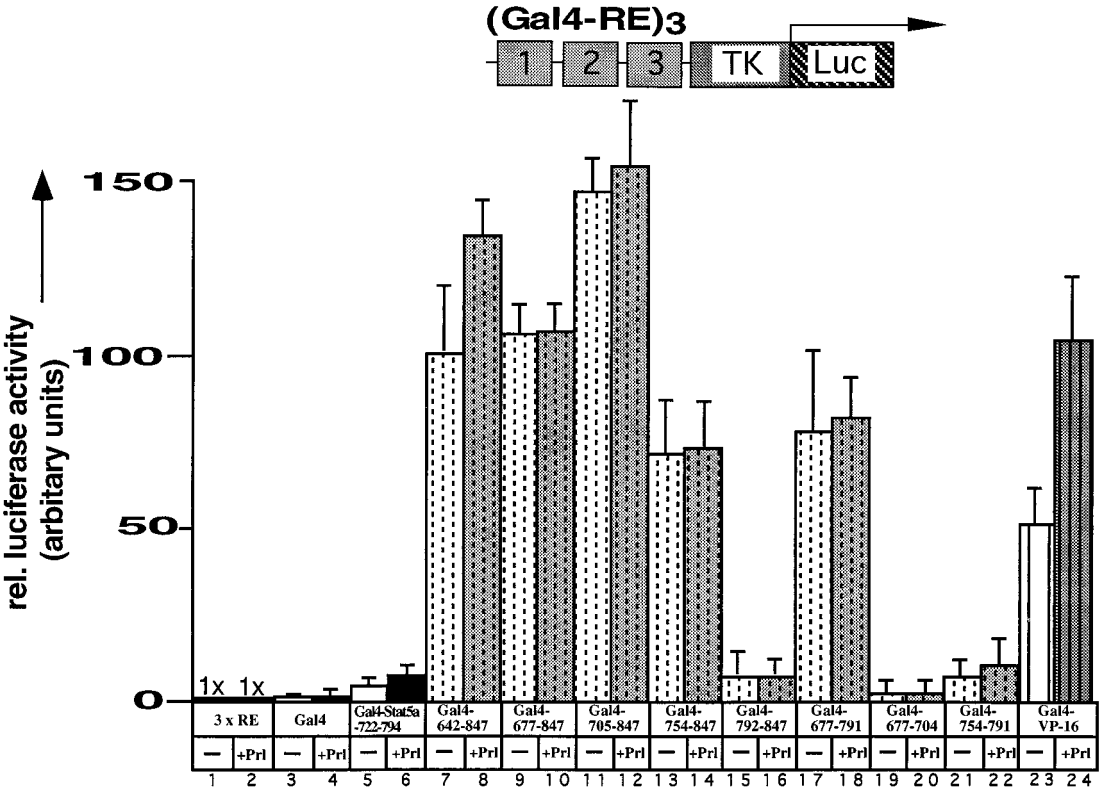


FIG. 5. Transactivation of a GAL4-luciferase construct by GAL4-Stat6 fusion proteins in Ba/F3-PR lymphocytes is not regulated by prolactin. The GAL4-Stat6 fusion genes indicated were introduced together with a GAL4-luciferase (Luc) reporter gene by electroporation into Ba/F3-PR cells. Transfected cells were treated with (+) or without (−) prolactin (Prl) for 20 h. Relative (rel.) luciferase activities were determined 24 h after electroporation. The luciferase activity of the GAL4 reporter construct [$(Gal4\text{-}RE)_3$] was considered 1. Four independent experiments were carried out.

siently transfected into Ba/F3-IL4R cells. Cells were kept in medium with 10% FCS and IL-4, cell extracts were prepared, and luciferase activities (Fig. 6C) were determined. The reporter construct and GAL4 showed no significant luciferase activity upon human IL-4 induction (Fig. 6C, lanes 2 and 4). Strong luciferase activity was obtained for GAL4-Stat6-677-791, and it was significantly upregulated upon IL-4 induction (Fig. 6C, lanes 5 and 6). In contrast, GAL4-VP16 showed no such upregulation upon IL-4 induction (Fig. 6C, lanes 7 and 8). These experiments (Fig. 6C) were repeated four times with consistent results. To further confirm IL-4-dependent regulation of the Stat6 transactivation domain in lymphoid cells, we also tested the constructs GAL4-642-847, GAL4-677-847, and GAL4-705-847 (Fig. 4A). When the activities in extracts from IL-4-treated and untreated cells were compared, we found that luciferase activity was enhanced upon cytokine treatment in cells transfected with constructs (data not shown).

Provision with the Stat6 transactivation domain enhances Stat5 induction of the β-casein, CIS, and OSM gene promoters. We recently mapped the Stat5 transactivation domain, and a region between amino acid positions 750 and 794 was found to be absolutely required for gene transcription (44). The results of experiments described above (Fig. 4 and 5) indicate that the transactivation domain of Stat5a is weaker than that of Stat6 when linked to a heterologous DNA binding domain. To compare the strength of the Stat5 and Stat6 transactivation domains within the context of a Stat molecule, we exchanged the transactivation domain of Stat5 with different parts of the transactivation domain of Stat6. The variant of Stat5a lacking the carboxyl-terminal 44 amino acids (Stat5aΔ750) served as the starting point. This molecule is not able to support transactivation by itself (44). Amino acids 677 to 847, 705 to 847, 754 to 847, 792 to 847, and 677 to 791 of Stat6 were fused to the carboxyl-terminal end of Stat5aΔ750. A fusion with the transactivation domain of VP16 (amino acids 411 to 489) served as a control (Fig. 7A).

COS7 cells were transiently transfected with the prolactin receptor, Stat5-Stat6 fusion constructs, and a β-casein gene promoter–luciferase construct. The expression of Stat5-Stat6 proteins was visualized by Western blotting analysis with a specific chicken antiserum directed against the carboxyl-terminal domain of Stat6 [Stat6 (633-837)] (Fig. 7B); tyrosine phosphorylation of Stat5-Stat6 proteins upon prolactin treatment of transfected cells was shown by Western blotting analysis with a phosphotyrosine-specific antibody (PY20) (Fig. 7B); specific DNA binding activity was observed in bandshift experiments (Fig. 7C, lanes 2, 5, 8, 11, and 14). Supershift experiments were carried out with Stat6-specific polyclonal antiserum (Fig. 7C, lanes 3, 6, 9, 12, and 15). All five Stat5-Stat6 fusion proteins can be tyrosine phosphorylated and acquire specific DNA binding activity upon prolactin treatment of transfected cells.

The transactivation potentials of Stat5a, Stat5aΔ750, and Stat5-Stat6 fusion proteins were measured with a β-casein gene promoter–luciferase construct. Wild-type Stat5a mediated a 15-fold increase in luciferase activity upon prolactin induction (Fig. 8A, lanes 1 and 2). Stat5aΔ750 was unable to support induction (Fig. 8A, lanes 3 and 4). Stat5aΔ750-Stat6-677-847 (Fig. 8A, lanes 5 and 6), Stat5aΔ750-Stat6-705-847 (lanes 7 and 8), Stat5aΔ750-Stat6-754-847 (lanes 9 and 10), and Stat5aΔ750-Stat6-677-791 (lanes 13 and 14) caused very

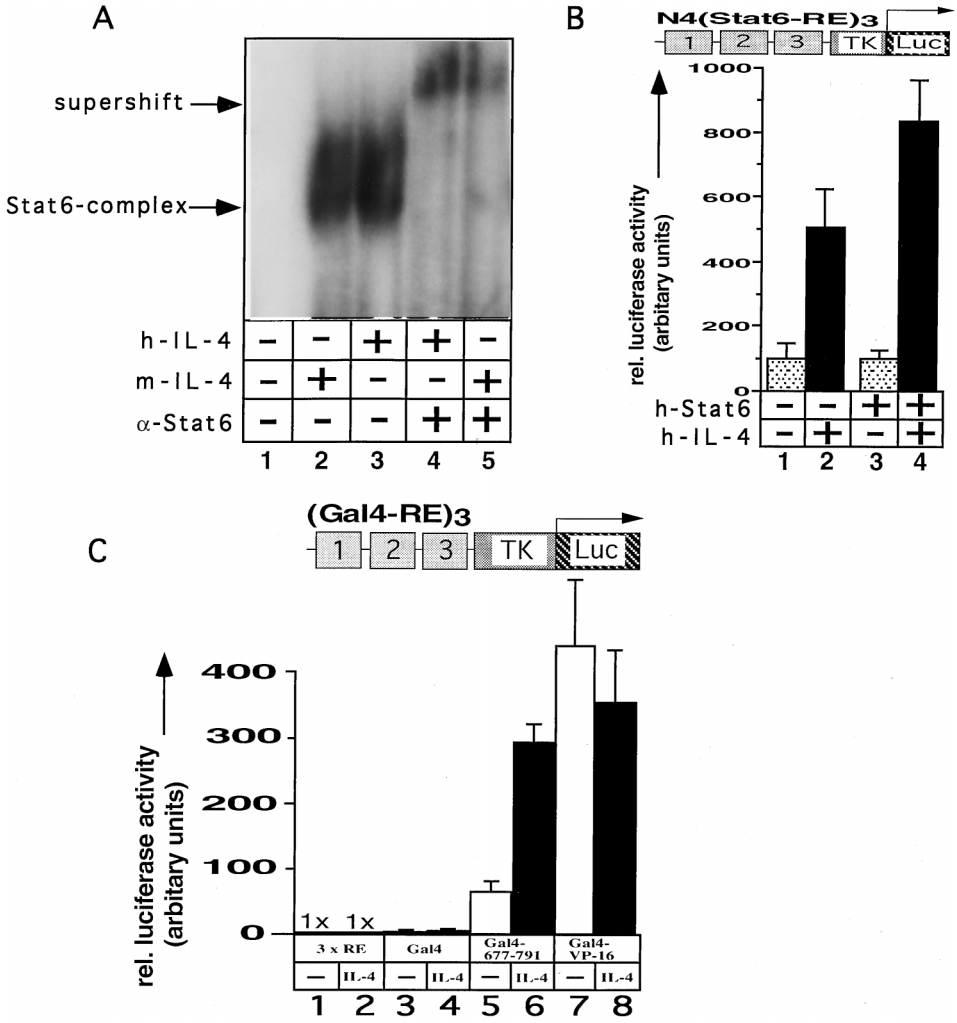


FIG. 6. Transactivation of a GAL4-luciferase construct by GAL4-Stat6 fusion proteins in Ba/F3-IL4R lymphocytes is regulated by IL-4. (A) DNA binding activities of murine Stat6 (m-Stat6) and h-Stat6. Whole-cell extracts were prepared and introduced into bandshift assays. The β-casein promoter Stat binding site was used as the probe (lanes 2 and 3). Supershift experiments were carried out with (+) or without (−) Stat6 (633 to 837)-specific antiserum (β-Stat6). (B) Transcriptional activation of Stat6 in Ba/F3-IL4R cells. Ba/F3-IL4R cells were transiently transfected with a reporter gene containing three copies of the Stat6 response element (immunoglobulin germ line heavy-chain ε promoter) [N4(Stat6-RE)$_3$] (lanes 1 to 4) and the h-Stat6 gene (lanes 3 and 4). Cells were stimulated with (+) human IL-4 for 20 h (lanes 2 and 4) or left unstimulated (−). Relative (rel.) luciferase activities were determined at 24 h after electroporation. Three independent experiments were carried out. (C) Ba/F3-IL4R cells were transiently transfected with the GAL4 fusion constructs indicated and a GAL4-luciferase reporter construct [(GAL4-RE)$_3$]. Transfected cells were treated with IL-4 for 20 h or left untreated (−). Relative (rel.) luciferase activities were determined at 24 h after electroporation. The luciferase activity of the GAL4-luciferase reporter construct in the absence of cotransfected factor constructs was considered 1. Four independent experiments were carried out.

strong induction upon prolactin activation. The induction was four to five times higher than that observed with the authentic Stat5 molecule. Slightly lower induction was observed with Stat5aΔ750-Stat6-792-847 (Fig. 8A, lanes 11 and 12), but it still exceeded that of wild-type Stat5.

We also determined the potentials of the transactivation domains of Stat5 and Stat6 by using a construct with multimerized Stat5 binding sites in its promoter region (Fig. 8B). The high-affinity proximal Stat site in the β-casein promoter, with the dyad Stat5 half sites separated by 3 nucleotides, was linked to the TK promoter and a luciferase gene [N3(Stat-RE)6-tk-luc] (44). The N3(Stat-RE)6-tk-luc reporter construct, the prolactin receptor, MGF-Stat5a, MGF-Stat5aΔ750, and Stat5-Stat6 fusion proteins were introduced into COS7 cells. Strong transcriptional activation upon prolactin treatment depended upon the transactivation domain of Stat6 (Fig. 8B, lanes 6, 8, and 10) or VP-16 (lane 14). Only weak or no transactivation was observed with MGF-Stat5a (Fig. 8B, lane 2), MGF-Stat5aΔ750 (lane 4), or Stat5aΔ750-Stat6-792-847 (lane 12). The transactivation potentials of Stat5-Stat6 fusion proteins confirm the results obtained with GAL4-Stat6 fusion proteins.

We observed that the Stat6 transactivation domain causes a higher induction of the β-casein gene promoter than the Stat5 transactivation domain (Fig. 8). To investigate whether this effect is restricted to the β-casein gene promoter or it extends to other target genes, we compared the induction of the CIS and OSM gene promoters (77, 78) achieved with Stat5 and Stat5aΔ750-Stat6-677-847. Transient transfections of MGF-Stat5a, Stat5aΔ750-Stat6-677-847, the prolactin receptor, and β-casein, CIS, and OSM gene promoter constructs were performed with COS7 cells; cell extracts were harvested, and luciferase activities were determined. For all three reporter constructs, the inducibility by the Stat5-Stat6 chimeric factor was

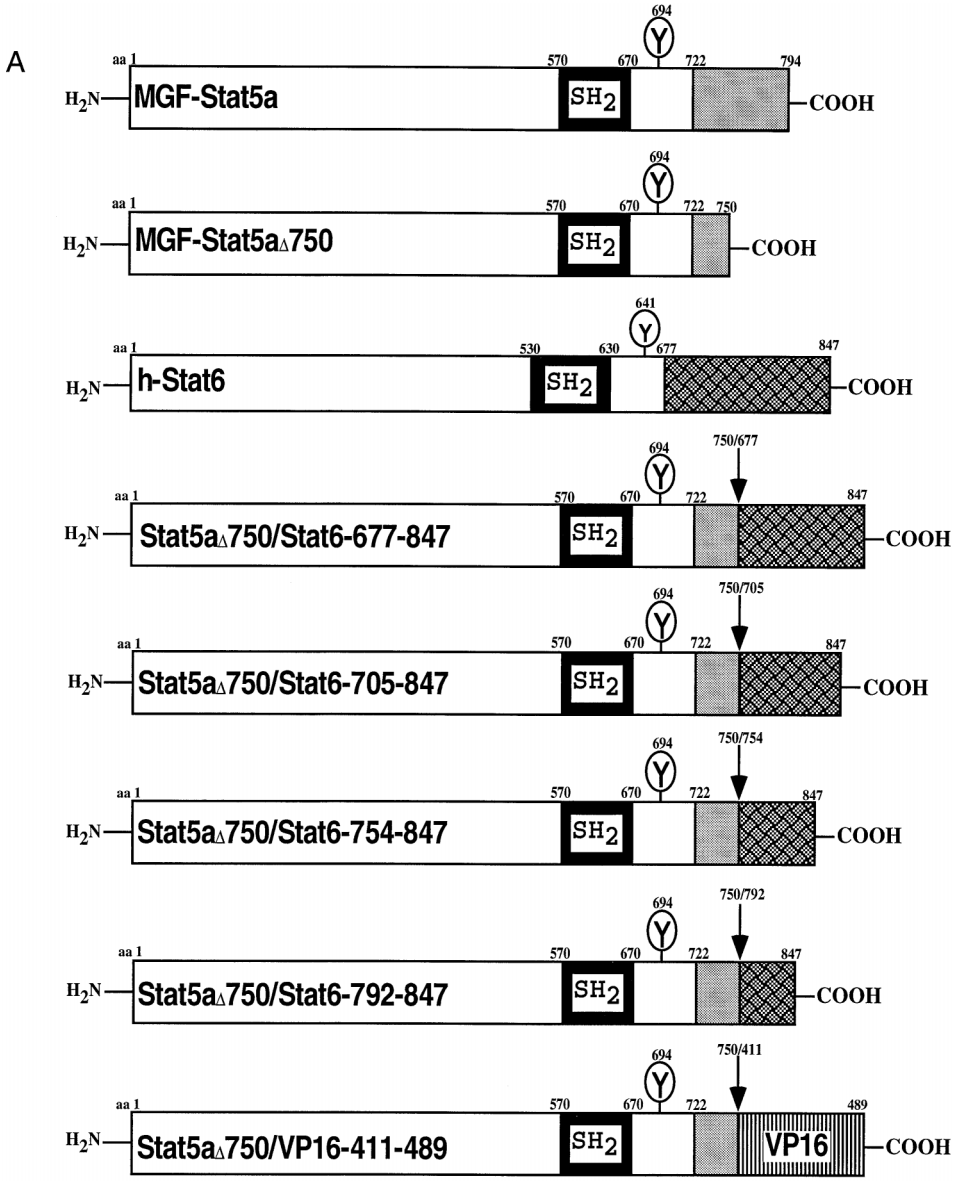


FIG. 7. Expression and activation of Stat5-Stat6 transactivation domain fusion proteins. (A) Structures of Stat5a, Stat5aΔ750 (lacking the Stat5 transactivation domain), Stat6, and Stat5-Stat6 transactivation domain fusion proteins. Amino acid (aa) positions, SH2 domains, and tyrosine residue 694 (Y), which is essential for tyrosine phosphorylation, are indicated. Arrows mark the junction between Stat5- and Stat6-specific sequences. Stat5aΔ750-VP16-411-489 contains amino acids 411 to 489 of the viral transactivator VP16. (B) Western blot analysis of Stat5-Stat6 transactivation domain fusion proteins. COS7 cells were transfected with the prolactin receptor, Stat5-Stat6 transactivation domain fusion constructs, and the β-galactosidase gene. Cells were left untreated (−) or treated with (+) prolactin for 1 h, and whole-cell extracts were prepared. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, blotted onto nitrocellulose, and incubated with a phosphotyrosine-specific antibody (α-PY20). The membrane was reprobed with a chicken antiserum specific for Stat6 (633-837) [α-Stat6(633-837)]. (C) DNA binding activities of Stat5-Stat6 transactivation domain fusion proteins. Whole-cell extracts were prepared as described for panel B. Bandshift experiments were performed. The proximal β-casein promoter Stat binding site served as the probe. A Stat6-specific antiserum was included in the binding reaction mixture in supershift experiments.

about fourfold higher than the induction observed with Stat5a (Fig. 9, lanes 2 and 4).

DISCUSSION

IL-4 is a cytokine important for the differentiation and proliferation of different cell types (8, 53, 54, 72). In human lymphocytes, IL-4 induction was shown to activate Jak1 and Jak3 (28, 39). IL-4 exerts part of its function through Stat6, a transcriptional activator. Stat6 was originally characterized as a specific mediator of IL-4-dependent gene transcription (32, 35, 61) but can also be activated by IL-13 (39).

Stat6 can be induced by IL-4 in lymphoid cells and cultured HC11 mammary epithelial cells. The biological role of the IL-4-mediated responses in mammary epithelial cells is not known. We observed, however, that in mammary epithelial cells, Stat6 causes the induction of β-casein gene expression. The β-casein gene promoter can also be induced by Stat6 in transfected COS7 cells. For transcriptional induction, the transactivation domain of Stat6, which is located within the

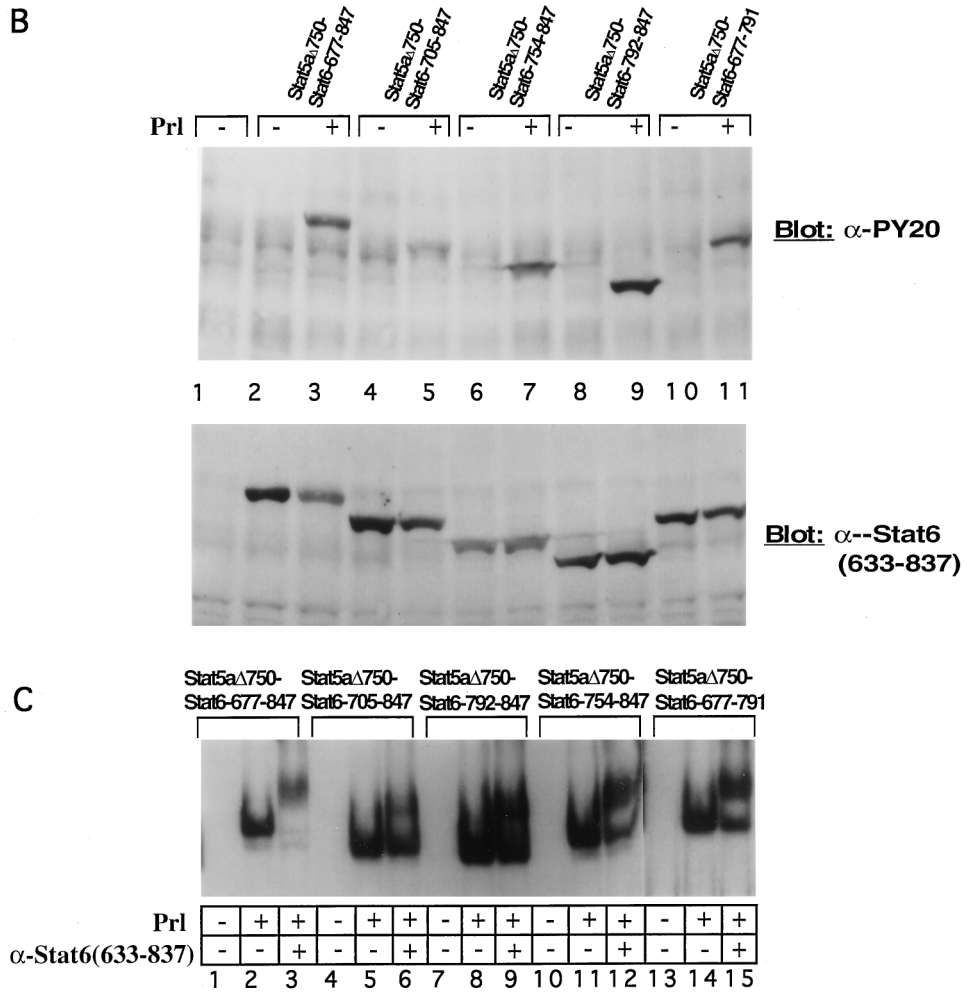


FIG. 7—*Continued.*

carboxyl-terminal 170 amino acids, is essential. This transactivation domain functions independently from the DNA binding domain of Stat6 and can be conferred to the heterologous GAL4 DNA binding domain or MGF-Stat5aΔ750. It is about five times more potent in lymphoid cells than in COS7 cells. Comparison of the strength of the transactivation domain of Stat5 with that of Stat6 in COS7 cells showed that the Stat6 domain is about 10 times more potent than is the Stat5 domain.

We have previously shown that efficient induction of the β-casein gene promoter in mammary epithelial cells is dependent on the synergistic action of Stat5 and the GR (68). These transcription factors form a molecular complex (68). Stat5a and Stat5b contain only a weak transactivation domain when tested on minimal promoters or as GAL4-Stat5 carboxyl-terminal fusion proteins (6, 44). It is conceivable that the strong transactivation domain of the GR ameliorates the action of Stat5. IL-4 induction of the β-casein gene promoter through Stat6 is as efficient as that through the combined action of prolactin and glucocorticoids. This might be due to the inherent strength of the transactivation domain of Stat6.

Cooperation with the GR is not restricted to Stat5. It was also observed with Stat6. Stat5 and Stat6 have the highest sequence homology among members of the Stat gene family. Since the carboxyl termini of Stat5 and Stat6 do not have significant sequence homologies, we suspect that other domains are involved in GR interactions. Extensive sequence homologies between Stat5 and Stat6 are found in the remainders of these molecules.

Mikita et al. also studied the transactivation potential of h-Stat6 (43). They showed that in vitro DNA binding of recombinant Stat6 is stronger to response elements with dyad half sites separated by 4 instead of 3 nucleotides. We found that the proximal β-casein promoter Stat site, with dyad half sites separated by 3 nucleotides, also efficiently bound Stat6 (Fig. 1C). Mikita et al. also showed that efficient gene transcription directed by the IL-4 response element derived from the immunoglobulin germ line heavy-chain ε promoter is dependent on C/EBP (43). Four C/EBP sites are present in the β-casein gene promoter region (11, 50). They did not observe transcriptional induction of multimerized Stat response elements without a C/EBP site linked to a TK minimal promoter and a luciferase gene. The promoter construct used in our study (Fig. 6B) retains a C/EBP site and inducibility. The contribution of C/EBP family members to Stat6-mediated gene transcription is complex, since multiple splice variants of the five C/EBP family members exist. Recent studies have revealed a role for C/EBP-α and C/EBP-δ in IL-4-induced gene transcription of the β-casein gene promoter in COS cells (81).

The domain structures and localization of the transactiva-

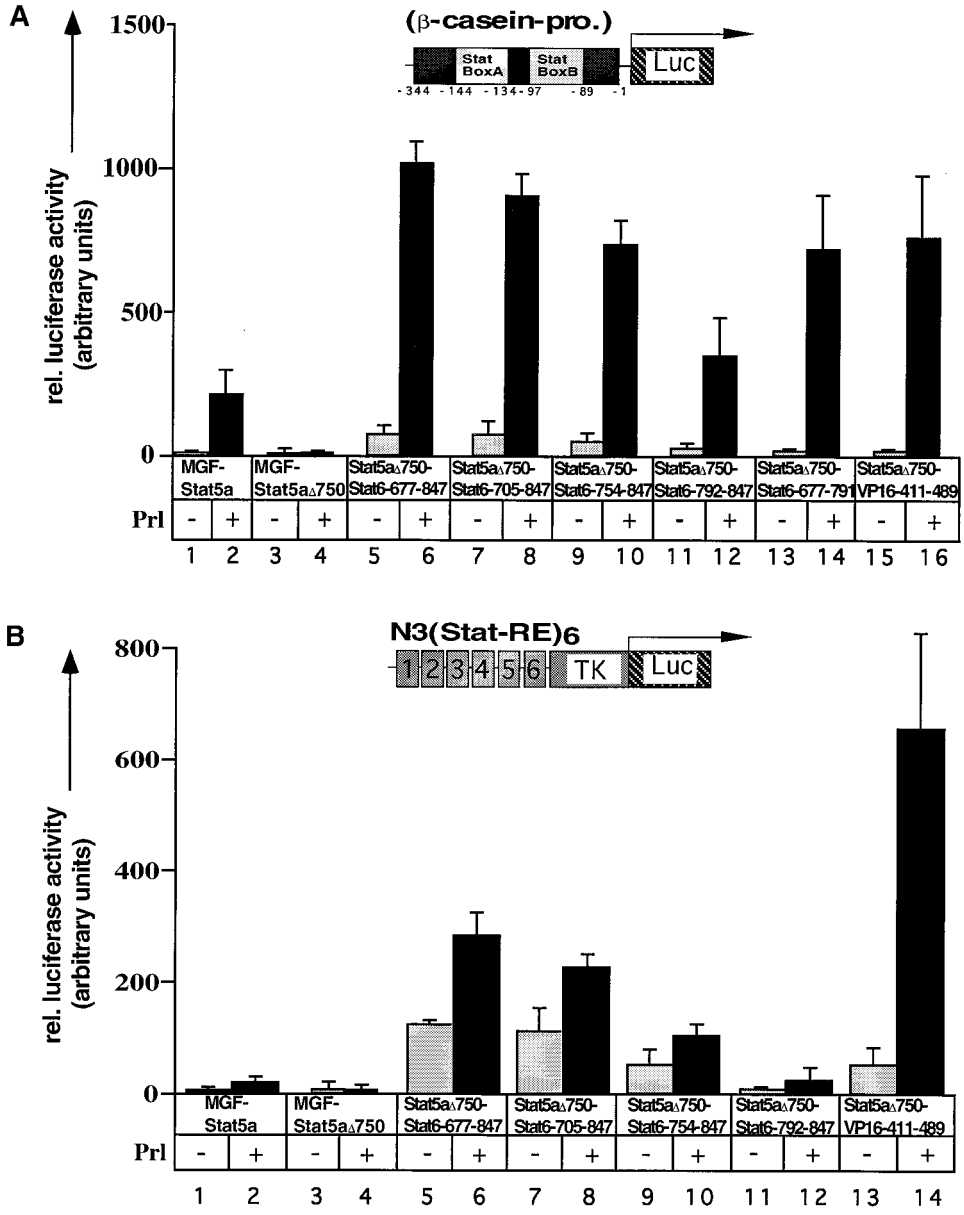


FIG. 8. Transactivation potentials of Stat5, Stat5Δ750, and Stat5-Stat6 transactivation domain fusion proteins. (A) COS7 cells were transfected with the β-casein gene promoter (β-casein-pro.)–luciferase (Luc) construct, the prolactin receptor, the β-galactosidase gene, and wild-type Stat5a (lanes 1 and 2), Stat5aΔ750 (lanes 3 and 4), Stat5aΔ750-Stat6-677-847 (lanes 5 and 6), Stat5aΔ750-Stat6-705-847 (lanes 7 and 8), Stat5aΔ750-Stat6-754-847 (lanes 9 and 10), Stat5aΔ750-Stat6-792-847 (lanes 11 and 12), Stat5aΔ750-Stat6-677-791 (lanes 13 and 14), or Stat5aΔ750-VP16-411-489 (lanes 15 and 16). Relative (rel.) luciferase activities were determined after 15 h of prolactin (Prl) treatment (+). −, untreated. (B) COS7 cells were transfected with the prolactin receptor, the β-galactosidase gene, wild-type Stat5a, Stat5Δ750, the Stat5-Stat6 transactivation domain fusion proteins indicated, and an N3(Stat-RE)$_6$ construct with six Stat5 binding sites linked to a *tk* minimal promoter and a luciferase gene. Relative luciferase activities were determined in extracts from unstimulated (−) and prolactin-induced (+) cells and normalized against β-galactosidase activity. Three independent experiments were carried out.

tion domains of Stat proteins have recently been investigated. The transactivation domains of Stat2, Stat3, Stat5a, and Stat5b were mapped to the carboxyl terminus (30, 44, 47, 49, 58). This is also the case for Stat6. Within this region, we find many proline, serine, threonine, leucine, and glutamine residues but not many acidic residues (Fig. 10). This is indicative of a non-acidic transactivation domain. Similar characteristics have been described for the transactivation domains of octamer factors and steroid hormone receptors (7, 27, 38). Our data based on GAL4 and Stat5aΔ750 fusion proteins indicate that a central core region (amino acids 677 to 791 [Fig. 10]) is important for transactivation.

We tested several carboxyl-terminal deletion mutants of Stat6 (Fig. 10). Stop codons were introduced at amino acids positions 677, 705, 754, and 792 of h-Stat6. These deletion mutants were analyzed for tyrosine phosphorylation, DNA binding, and transactivation potential upon IL-4 induction. All variants were tyrosine phosphorylated and able to specifically bind DNA (data not shown). Only the deletion mutant h-Stat6Δ677 lacked the ability to transactivate efficiently. This

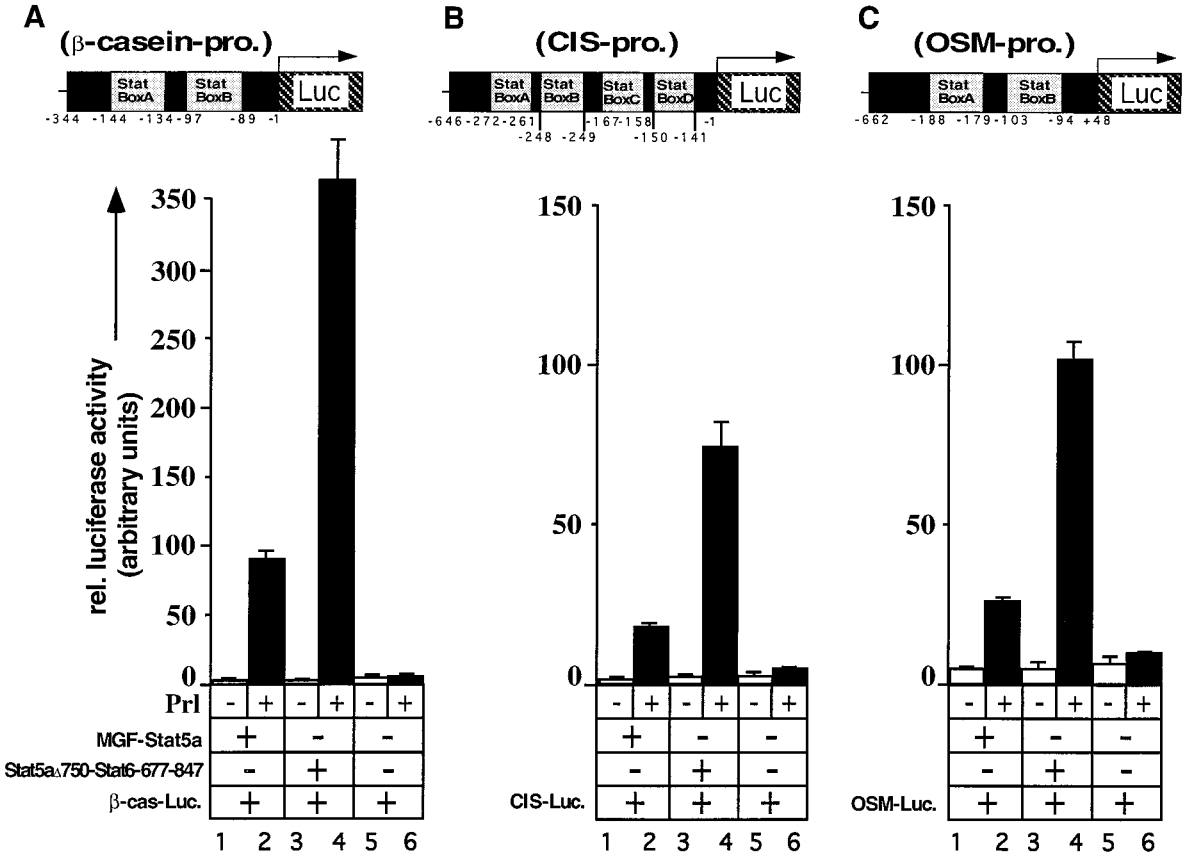


FIG. 9. Comparison of the Stat5 and Stat6 transactivation domains and their effects on β-casein (β-cas) (A), CIS (B), and OSM (C) gene promoters (pro.). COS7 cells were transiently transfected with MGF-Stat5a, Stat5aΔ750-Stat6-677-847, the prolactin receptor, and the reporter constructs indicated. The numbering refers to the transcription initiation sites in individual genes. The positions of Stat boxes are shown. The β-galactosidase gene was included to monitor transfection efficiency. Transfected cells were left untreated (−) or treated (+) with prolactin (Prl), cell extracts were prepared, and relative (rel.) luciferase (Luc.) activities were determined. The data obtained were normalized against β-galactosidase activities. Three individual experiments were carried out.

deletion mutant is similar to h-Stat6Δ661 (43). It is a potent dominant negative variant and suppresses the activity of endogenous Stat6 in HepG2 cells activated by IL-4.

Recently, we observed that the carboxyl terminus of Stat5 is important for downregulation of DNA binding by tyrosine dephosphorylation (44). Therefore, Stat6 deletion mutants were also analyzed for their DNA binding kinetics after IL-4 activation. Consistent with the observations made with Stat5 variants, a deletion of 93, 142, or 170 amino acids of h-Stat6 resulted in impaired downregulation and prolonged tyrosine phosphorylation (data not shown). We also observed a delay in the downregulation of DNA binding with constructs Stat 5aΔ750 and Stat5aΔ750-Stat6-792-847. Constructs Stat5aΔ 750-Stat6-677-847, Stat5aΔ750-Stat6-705-847, Stat5aΔ750-Stat 6-677-847, and Stat5aΔ750-Stat6-677-791 showed wild-type behavior (data not shown). From the results of these experiments, we conclude that the region between amino acids 754 and 792 of h-Stat6 is important for the downregulation of DNA binding through tyrosine dephosphorylation.

A remarkable feature of the Stat6 transactivation domain is the cell type dependence of its efficiency. The GAL4-Stat6 fusion was active in both COS7 and Ba/F3 cells but was much stronger in lymphoid cells. Studies of the interactions of the Stat6 transactivation domain with cell type-specific coactivators, corepressors, and components of the basal transcription initiation complex might be interesting in this context.

A second interesting feature of the Stat6 transactivation domain concerns autonomous regulation through IL-4. Although GAL4-Stat6 transactivation domain constructs exhibit high constitutive activities, they still can be enhanced by the action of IL-4. The transactivation domain is rich in serine and threonine residues. It is conceivable that the enhanced activity of the Stat6 transactivation domain upon IL-4 stimulation is correlated with phosphorylation events in this region. Mitogen-activated protein kinase activity was shown to be increased after IL-4R activation in human keratinocytes (75). Serine or threonine phosphorylation has been implicated in the formation of Stat1-, Stat3-, and Stat5-DNA complexes (5, 74, 80). Phosphorylation of Stat1 and Stat3 was ascribed to the action of mitogen-activated protein kinase (74, 80). This enzyme does not seem to be responsible for Stat5 phosphorylation upon cytokine activation (5, 73). No cytokine dependence of the Stat5 transactivation domain fused to the DNA binding domain of GAL4 in Ba/F3-PR cells upon prolactin or interleukin-3 activation was observed (data not shown).

It is interesting that efficient transcription from the β-casein promoter was not observed in Ba/F3 cells stimulated with prolactin, IL-3, or IL-4 (data not shown), indicating that Stat5 or Stat6 activation is not a sufficient requirement for transcriptional induction. This is different from an artificial construct which contains multimerized Stat binding sites in its promoter region (44) (data not shown). The β-casein gene promoter has a modular design and contains multiple transcription factor binding sites in addition to the Stat sites (14, 18, 57, 64).

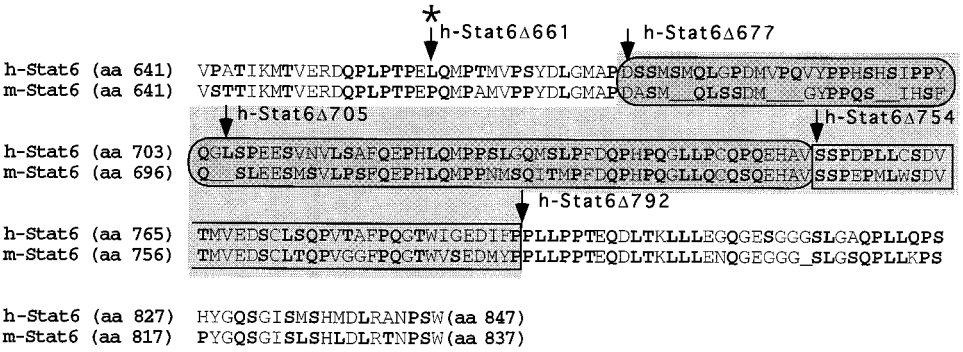


FIG. 10. Sequence alignment of the carboxyl-terminal transactivation domains of murine (m-Stat6) (48) and h-Stat6 (24). Proline, serine, threonine, leucine, and glutamine amino acids are in boldface. They could be important for the nonacidic transactivation domains. The shaded area (amino acid [aa] positions 677 to 792) is the minimal transactivation domain of h-Stat6 defined here. The region from amino acids 677 to 754 was found to be crucial for IL-4-mediated regulation of the transactivation domain. The region from amino acids 754 to 792 of h-Stat6 was found to be crucial for the downregulation of activated Stat6. Arrows indicate the positions of the truncation variants analyzed here. The asterisk indicates the truncated h-Stat6 variant described by Mikita et al. (43). The accession numbers of sequences and the programs used for alignment are described in Materials and Methods.

CCAAT family members also were shown to bind to multiple sites within the β-casein gene promoter close to the two Stat sites (11, 50). It is possible that repressor sites bind factors like C/EBP-β, C/EBP-γ, YY1, and SBP within the promoter region of the β-casein gene promoter (1, 2, 42). These repressors might not be relieved in lymphoid cells, superimposing an additional layer of regulation to the Jak-Stat pathway.

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